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Award Number: W81XWH-04-1-0301

TITLE: Exploiting Novel Calcium-Mediated Apoptotic Processes for the Treatment of Human Breast Cancers with Elevated NQO1 Levels

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REPORT DATE: March 2005

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	March 2005	Annual Summary (6 Feb 2004 - 5 Feb 2005)

4. TITLE AND SUBTITLE
Exploiting Novel Calcium-Mediated Apoptotic Processes for the Treatment of Human Breast Cancers with Elevated NQO1 Levels

5. FUNDING NUMBERS
W81XWH-04-1-0301

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

Alterations in the regulation and initiation of cell death, in particular caspase-mediated apoptotic cell death, have been associated with a vast array of pathological disease states such as cancer, and the development of resistance to cancer chemotherapies. β-Lapachone (β-Lap), a naturally occurring 1,2 naphthoquinone, has been shown by our laboratory to induce non-caspase-mediated apoptosis in breast and prostate cancer cells that express the two electron oxidoreductase, NAD(P)H:quinone oxidoreductase (NQO1). Here, we demonstrate that poly(ADP)-ribose polymerase (PARP) hyperactivation, occurs shortly after β-lap exposure in an NQO1 dependent manner. Hyperactivation of PARP is consistent with the presence of DNA strand breaks detected by comet and γH2AX formation. Chemical inhibition of PARP activity in MCF-7 human breast cancer cells by 3-aminobenzamide or DPQ suppressed β-lap-induced apoptosis consistent with a role for PARP hyperactivation in β-lap induced cell death. Chelation of intracellular Ca2+ using BATPA-AM after β-lap treatment abrogated γH2AX formation, PARP activation, and dramatically blocked β-lap induced death as measured by long-term growth assays. Collectively, these data demonstrate the importance of Ca2+-mediated PARP hyperactivation in β-lap-induced cell death. We will discuss how this Ca2+-dependent cell death can be exploited to effectively target and treat tumors that have lost their ability to undergo caspase-mediated apoptosis.

14. SUBJECT TERMS
Intracellular calcium, apoptosis, b-lapachone, breast cancer, Poly(ADP)-ribose polymerase, therapeutics, NQO1

15. NUMBER OF PAGES
8

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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Introduction and Summary

ß-Lapachone (ß-Lap) is a naturally occurring 1,2 napthoquinone present in the bark of the South American lapacho tree. Our lab has shown that ß-lap induced-apoptosis is enhanced in breast and prostate cancer cells that express the two electron oxidoreductase NAD(P)H:quinone oxidoreductase (NQO1, E.C.1.6.99.2) enzymatic activity. ß-Lap-induced apoptosis was unique in that an ~60 kDa PARP cleavage fragment as well as atypical p53 were observed consistent with the activation calcium (Ca^{2+})-dependent protease μ-calpain (Pink et al. 2000b; Tagliarino et al. 2003). In addition, ß-lap was found to elicit a rise in intracellular calcium, due to its release from endoplasmic reticulum (ER) Ca^{2+} stores shortly after drug administration. Treatment of cells with BAPTA-AM, an intracellular Ca^{2+} chelator, prior to ß-lap exposure could modulate apoptotic signaling but not significantly alter death (Tagliarino et al. 2001). Collectively these data supported a critical but not sufficient role for Ca^{2+} in NQO1 mediated ß-Lap-induced programmed cell death (PCD).

To explore a possible connection between Ca^{2+} release and PARP activation NQO1 proficient MCF-7 cells were exposed to ß-lap and the effects of an increase in cytosolic Ca^{2+} were investigated. Buffering the rise in intracellular Ca^{2+} after ß-lap treatment using BAPTA-AM prevented death, and proteolysis in NQO1 positive MCF-7 cells. Furthermore, short exposures of ß-lap caused an increase in poly (ADP)-ribosylated (PAR) proteins, H2AX phosphorylation, and comet formation, which was found to be Ca^{2+}-dependent. Furthermore, inhibition of PARP using the inhibitors 3-aminobenzamide (3-AB) and DPQ could abrogate ß-lap-induced apoptosis. Based on these new findings, we now report a critical role of Ca^{2+} in the induction of PARP activation that is intimately involved in ß-lap programmed cell death. Collectively, these data suggest that ß-lap would be an effective cancer chemotherapeutic agent that has the potential for effectively targeting and treating breast cancer tumors, which have lost their ability to undergo caspase-mediated apoptosis.

Body

ß-Lapachone-induced cancer cell death and proteolysis is time and calcium dependent

ß-Lap has been previously shown to be an effective chemotherapeutic agent in human cancer cell lines that have NQO1 enzymatic activity (Pink et al. 2000a). Therefore, to ascertain whether there is a dose and time relationship for effective ß-lap treatment, log-phase MCF-7 breast cancer epithelial cells, which are high in NQO1 enzymatic activity, were tested for their sensitivity to ß-Lap at various exposure times and doses. ß-Lap effectively killed MCF-7 cells according to relative survival assays at doses greater than 4 μM for 2h, however lower doses ≤ 3 μM required longer exposure times to elicit cell death. Furthermore, our lab has demonstrated that ß-lap-induced cell death in cancer cell lines was unique, resulting in PARP and p53 proteolysis that was distinct from those caused by caspase activating agents (Pink et al. 2000b). Treatment with ß-lap resulted in an ~60-kDa PARP cleavage fragment and intracellular cleavage of p53 in NQO1 expressing breast cancer cells. MCF-7 cells treated with staurosporine exhibited an 89 kDa PARP cleavage fragment consistent with caspase activation (Bertrand et al. 1994; Koh et al. 1995; Zhang et al. 1996). PARP cleavage could be prevented after staurosporine exposure upon co-treatment with the pan-caspase inhibitor zVAD-fmk. However, co-treatment of zVAD-fmk and ß-lap did not prevent ß-lap-induced atypical PARP or p53 cleavage. This is consistent with previous data that ß-Lap-induced cleavage events were attributable to the activation of the Ca^{2+}-dependent protease μ-calpain (Tagliarino et al. 2003). Furthermore, pre-treatment with the intracellular calcium chelator BAPTA-AM, at the minimum dose/time to commitment of the death (e.g. 4 μM ß-lap; 2h drug

exposure) could abrogate ß-lap lethality and atypical p53 and PARP proteolysis between 20 and 24h after drug exposure. In addition, BAPTA-AM protection is similar to what is observed with co-treatment of the NQO1 inhibitor dicoumarol, which competes with NADH or NADPH for binding to the oxidized form of the enzyme thereby preventing reduction of quinines (Hosoda et al. 1974; Hollander and Ernster 1975) illustrating the importance of calcium in ß-lap cell death. Previous studies have confirmed that BAPTA did not alter NQO1 enzymatic activity using standard enzymatic assays (Tagliarino et al. 2001). These findings indicate that short ß-lap exposures elicit non-caspase calcium-meditated cell death and proteolysis.

ß-Lap treatment causes a PARP-mediated cell death

A dramatic depletion in ATP pools is seen shortly after ß-lap treatment, and this nucleotide loss was found to be Ca^{2+} dependent (Tagliarino et al. 2001). NAD^+ loss as a consequence of PARP activation has also been observed to contribute to cell death after ß-lap treatment in NQO1 deficient cells (Liu et al. 2002). PARP functions as a surveyor of the genome. In response to DNA breaks, PARP is immediately activated and catalyzes the conversion of ß-nicotinamide adenine dinucleotide (NAD) into linear or multibranched poly(ADP-ribose) polymers on various proteins usually associated with chromatin and other nuclear acceptor proteins (e.g. PARP itself, XRCC1, histones etc) (Ame et al. 2004). This post-translational modification helps to facilitate the repair process by opening the DNA structure and recruiting DNA repair enzymes to the site of the break. In cases of severe DNA damage, such as ischemia reperfusion or high dose MNNG exposure PARP hyperactivation can cause cellular depletion of NAD^+ leading to either necrotic or apoptotic non-caspase-AIF-mediated cell death (Szabo and Dawson 1998; Yu et al. 2002). However to date, it is unclear how NQO1 status and calcium signaling may mediate PARP activation in ß-lap induced PCD. Therefore, to test the relationship of PARP activation to NQO1 status, isogenic MDA-MB-231 cells that were previously transfected with human NQO1 were used (Pink et al. 2000a). Cells proficient in NQO1 enzymatic activity showed much higher PARP activation than their NQO1 negative counterparts as indicated by the increase in poly(ADP-ribose) polymers (PAR) formed after ß-lap treatment. Treatment of NQO1 deficient cells at ß-lap doses $>LD_{50}$ value of 6.6 μM $\pm$ 1.4 (Reinicke et al. unpublished results), was not sufficient to cause notable PAR accumulation. Furthermore, PARP activation after ß-lap treatment could be suppressed by pre-treatment with BAPTA-AM, which abrogated the formation of PAR even at high doses. These results were confirmed by confocal microscopy using a monoclonal antibody to PAR. PARP activation was seen 10' after 4 μM ß-lap exposure, as illustrated by the accumulation of PAR-modified proteins in the nucleus, with most robust poly (ADP-ribosyl)ation occurring 30' after drug exposure. This increase in PAR formation dissipates 90' post-drug treatment corresponding with the loss of ATP in the cells (Tagliarino et al. 2001).

To determine if NQO1-associated PARP activation contributes to ß-lap cell death MCF-7 cells were treated with the classical PARP inhibitor 3-aminobenzamide (3-AB). 3-AB is a nicotinamide analog and acts as a competitive inhibitor of PARP activation. Co-treatment with 3-AB or a more potent PARP inhibitor DPQ during ß-lap treatment prevented ß-lap-induced cell death as ascertained by the TUNEL assay in $NQO1^+$ cells at a lethal dose of 5 μM ß-lap. It is important to note that the results obtained using 3-AB were not attributable with inhibition of NQO1 enzymatic activity. 3-AB doses >100 fold greater than those used in relative survival assays using ß-lap or menadione as a substrate did not alter NQO1 activity in *in vitro*

enzymatic activity assays. Taken together, these findings indicate that Ca^{2+}-induced PARP hyperactivation is necessary for ß-Lap-induced cell death in NQO1 proficient cells.

H2AX phosphorylation occurs after ß-lap exposure

PARP activation has been well documented to be a result of DNA insult resulting, usually in DNA single strand breaks (Miller 1975; Smulson et al. 1975; Smulson et al. 1977). H2AX is a variant of one of the core histones, H2A, which is unique because it contains a short COOH terminal tail not found in other mammalian H2A isoforms (Mannironi et al. 1989). H2AX is distinct due to a highly conserved serine residue (ser139) located 4 amino acids from the COOH terminus, which is rapidly phosphorylated upon DNA damage (Rogakou et al. 1998). Once the DNA damage response has been activated members of the phosphotidylinositol-3 kinase-like family of kinases (PIKK) can be activated (Shiloh 2003). Three of these PIKK members, ataxia telangiectacisia mutated (ATM), ATM-and Rad3-related (ATR), and DNA dependent protein kinase (DNA-PK) mediate signal transduction at the site of the break and can immediately phosphorylate H2AX (Burma et al. 2001; Fernandez-Capetillo et al. 2002; Brown and Baltimore 2003). Therefore, to ascertain whether PARP activation is a direct result of ß-Lap-induced DNA damage H2AXγ levels were examined. MCF-7 and MDA-MB-231 cells were treated with 4 or 5 μM ß-lap and cells were harvested for western blot analyses at various times during drug treatment and probed for H2AXγ. Surprisingly, H2AX phosphorylation occurs 30' after ß-lap treatment. These results were confirmed by confocal microscopy. MCF-7 cells treated with ß-lap showed a time dependent appearance of H2AXγ foci. Furthermore, ß-lap-induced foci were similar to that induced by 5 Gy IR. These results were confirmed by the presence of comet formation after ß-lap treatment. Interestingly, ß-lap-induced H2AX phosphorylation can be partially abrogated by BAPTA-AM pre-treatment. This is distinct from IR-induced strand breaks, where BAPTA-AM had no effect on H2AXγ status. These data suggest that inhibition of H2AXγ by BAPTA-AM after ß-lap treatment is not due to Mg^{2+} chelation, which may affect PIKK function. In addition, the kinetics of H2AX phosphorylation are similar to the kinetic of PARP activation. Both H2AXγ and PAR are seen at 30' following ß-lap exposure. In addition BAPTA-AM loading prevents PAR formation and partially abrogates H2AXγ after ß-lap treatment. Collectively these data suggest a role for Ca^{2+} in the modulation of nuclear signaling events that lead to PARP hyperactivation and H2AX phosphorylation.

Key Accomplishments

- Creation of stable $PARP^{+/+}$ and $PARP^{-/-}$ MEF cells lines stably expressing active human NQO1 protein
- Creation of stable knock down (KD) PARP-1 protein using shPARP in human breast cancer cell lines MCF-7, MDA-MB-231 $NQO1^{+}$ and MDA-MB-231 $NQO1^{-}$
- Evaluation of time course of PARP activation after β-lapachone treatment in breast cancer cell lines with proficient and KD PARP protein levels and $PARP^{-/-}$ MEF ± hNQO1 using PARP activity blots
- Examination of the following endpoints after ß-lap treatment ±BAPTA-AM, ±PARP inhibitors (3-AB and DPQ) in MCF-7 and MCF-7 cells with KD PARP-1: apoptotic time course using the TUNEL assay, cell survival using long term growth assays, and apoptotic proteolysis via western blot.
- Examination of cell survival in long term growth assays in $PARP^{+/+}$ and $PARP^{-/-}$ $hNQO1^{+}$ and $hNQO1^{-}$ cells

Reportable Outcomes

The following reportable outcomes that have resulted from this award are as follows:

1. Successful completion of graduate school coursework
2. Passed qualifying examinations I and II; advancement to candidacy
3. Presentation at the Case Western Reserve University 2004 Department of Pharmacology Retreat
4. Invited Speaker, The University of Toledo, 2004
5. Presentation at Case Western Reserve University Biomedical Graduate Student Symposium, 2004 and 2005
6. Submitted abstract to the DOD Era of Hope Conference, 2005
7. Creation of $PARP^{+/+}$ and $PARP^{-/-}$ mouse embryonic fibroblasts with human NQO1 using a retroviral vector
8. Development of stable breast cancer cell lines MCF-7, MDA-MB-231 $NQO1^{+}$ and MDA-MB-231 $NQO1^{-}$ with knock down PARP-1 expression using RNAi.
9. Department of Energy sponsored trip to attend the 54^{th} Meeting of the Nobel Laureates, Lindau Germany

Conclusions

Here, we demonstrate that PARP hyperactivation, occurs shortly after β-lap exposure in an NQO1 dependent manner. Hyperactivation of PARP is consistent with the presence of DNA strand breaks detected by comet and γH2AX formation. Chemical inhibition of PARP activity in MCF-7 human breast cancer cells by 3-aminobenzamide or DPQ suppressed β-lap-induced apoptosis consistent with a role for PARP hyperactivation in β-lap induced cell death. Chelation of intracellular Ca2+ using BATPA-AM after β-lap treatment partially abrogated γH2AX, but not comet formation. In addition BAPTA-AM dramatically blocked both PARP activation and β-lap induced death as measured by long-term growth assays. Collectively, these data demonstrate the importance of Ca2+ mediated-PARP hyperactivation in β-lap-induced cell death. We will discuss how this Ca2+-dependent cell death can be exploited to effectively target and treat tumors that have lost their ability to undergo caspase-mediated apoptosis.

References

Ame, J.C., C. Spenlehauer, and G. de Murcia. 2004. The PARP superfamily. *Bioessays* **26**: 882-93.

Bertrand, R., E. Solary, P. O'Connor, K.W. Kohn, and Y. Pommier. 1994. Induction of a common pathway of apoptosis by staurosporine. *Exp Cell Res* **211**: 314-21.

Boothman, D.A. and A.B. Pardee. 1989. Inhibition of radiation-induced neoplastic transformation by beta-lapachone. *Proc Natl Acad Sci U S A* **86**: 4963-7.

Brown, E.J. and D. Baltimore. 2003. Essential and dispensable roles of ATR in cell cycle arrest and genome maintenance. *Genes Dev* **17**: 615-28.

Burma, S., B.P. Chen, M. Murphy, A. Kurimasa, and D.J. Chen. 2001. ATM phosphorylates histone H2AX in response to DNA double-strand breaks. *J Biol Chem* **276**: 42462-7.

Fernandez-Capetillo, O., H.T. Chen, A. Celeste, I. Ward, P.J. Romanienko, J.C. Morales, K. Naka, Z. Xia, R.D. Camerini-Otero, N. Motoyama, P.B. Carpenter, W.M. Bonner, J. Chen, and A. Nussenzweig. 2002. DNA damage-induced G2-M checkpoint activation by histone H2AX and 53BP1. *Nat Cell Biol* **4**: 993-7.

Hollander, P.M. and L. Ernster. 1975. Studies on the reaction mechanism of DT diaphorase. Action of dead-end inhibitors and effects of phospholipids. *Arch Biochem Biophys* **169**: 560-7.

Hosoda, S., W. Nakamura, and K. Hayashi. 1974. Properties and reaction mechanism of DT diaphorase from rat liver. *J Biol Chem* **249**: 6416-23.

Koh, J.Y., M.B. Wie, B.J. Gwag, S.L. Sensi, L.M. Canzoniero, J. Demaro, C. Csernansky, and D.W. Choi. 1995. Staurosporine-induced neuronal apoptosis. *Exp Neurol* **135**: 153-9.

Liu, T.J., S.Y. Lin, and Y.P. Chau. 2002. Inhibition of poly(ADP-ribose) polymerase activation attenuates beta-lapachone-induced necrotic cell death in human osteosarcoma cells. *Toxicol Appl Pharmacol* **182**: 116-25.

Mannironi, C., W.M. Bonner, and C.L. Hatch. 1989. H2A.X. a histone isoprotein with a conserved C-terminal sequence, is encoded by a novel mRNA with both DNA replication type and polyA 3' processing signals. *Nucleic Acids Res* **17**: 9113-26.

Miller, E.G. 1975. Effect of deoxyribonuclease I on the number and length of chains of poly(ADP-ribose) synthesized, in vitro. *Biochem Biophys Res Commun* **66**: 280-6.

Pink, J.J., S.M. Planchon, C. Tagliarino, M.E. Varnes, D. Siegel, and D.A. Boothman. 2000a. NAD(P)H:Quinone oxidoreductase activity is the principal determinant of beta-lapachone cytotoxicity. *J Biol Chem* **275**: 5416-24.

Pink, J.J., S. Wuerzberger-Davis, C. Tagliarino, S.M. Planchon, X. Yang, C.J. Froelich, and D.A. Boothman. 2000b. Activation of a cysteine protease in MCF-7 and T47D breast cancer cells during beta-lapachone-mediated apoptosis. *Exp Cell Res* **255**: 144-55.

Rogakou, E.P., D.R. Pilch, A.H. Orr, V.S. Ivanova, and W.M. Bonner. 1998. DNA double-stranded breaks induce histone H2AX phosphorylation on serine 139. *J Biol Chem* **273**: 5858-68.

Shiloh, Y. 2003. ATM and related protein kinases: safeguarding genome integrity. *Nat Rev Cancer* **3**: 155-68.

Smulson, M., P. Stark, M. Gazzoli, and J. Roberts. 1975. Release of template restriction for DNA synthesis by poly ADP(ribose) polymerase during the HeLa cell cycle. *Exp Cell Res* **90**: 175-82.

Smulson, M.E., P. Schein, D.W. Mullins, Jr., and S. Sudhakar. 1977. A putative role for nicotinamide adenine dinucleotide-promoted nuclear protein modification in the antitumor activity of N-methyl-N-nitrosourea. *Cancer Res* **37**: 3006-12.

Szabo, C. and V.L. Dawson. 1998. Role of poly(ADP-ribose) synthetase in inflammation and ischaemia-reperfusion. *Trends Pharmacol Sci* **19**: 287-98.

Tagliarino, C., J.J. Pink, G.R. Dubyak, A.L. Nieminen, and D.A. Boothman. 2001. Calcium is a key signaling molecule in beta-lapachone-mediated cell death. *J Biol Chem* **276**: 19150-9.

Tagliarino, C., J.J. Pink, K.E. Reinicke, S.M. Simmers, S.M. Wuerzberger-Davis, and D.A. Boothman. 2003. Mu-calpain activation in beta-lapachone-mediated apoptosis. *Cancer Biol Ther* **2**: 141-52.

Yu, S.W., H. Wang, M.F. Poitras, C. Coombs, W.J. Bowers, H.J. Federoff, G.G. Poirier, T.M. Dawson, and V.L. Dawson. 2002. Mediation of poly(ADP-ribose) polymerase-1-dependent cell death by apoptosis-inducing factor. *Science* **297**: 259-63.

Zhang, H., T. Hoang, B. Saeed, and S.C. Ng. 1996. Induction of apoptosis in prostatic tumor cell line DU145 by staurosporine, a potent inhibitor of protein kinases. *Prostate* **29**: 69-76.